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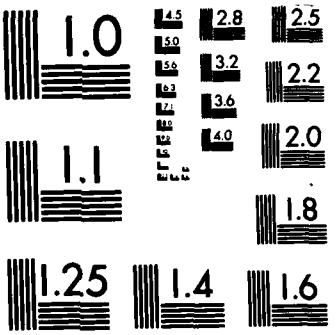

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
Attempts are being made to modify or destroy the Rh (D) antigen under conditions which will produce Rh negative erythrocytes of transfusable quality. To this end the effects of various lipase and membrane perturbants are being examined. Isolation and biochemical characterization of the D antigen is also being undertaken in order to gather information which will help formulate ways of modifying its expression. Originator Supplied Key Words Include.

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Our overall goal is to produce Rh_0 (D) negative erythrocytes which can be used in transfusion therapy. We report here our initial attempts to develop procedures for the removal or inactivation of the D antigen and the determination of its biochemical make up.

Phospholipase A Treatment

Incubation of intact erythrocytes with phospholipases of the A_2 type result in the degradation of membrane phosphatidylcholine to lysophosphatidylcholine and long chain fatty acids both of which remain bound to the cell membrane. It has been previously reported that treatment of red cells with phospholipase A_2 from bee venom produces a loss of D antigen activity as high as 50% of the original.

We have surveyed the effect of various phospholipase A_2 enzymes upon D antigen expression. Incubation of D+ cells with bee venom phospholipase in phosphate buffered saline containing K, Ca and Mg ions at pH's 7.4 or 8.0 and 37° results in a decrease in hemagglutination titer with undiluted human anti D antiserum of from 3+ to 1-2+ and a complete loss of agglutination of treated cells at a 1:2 dilution of antiserum. No further decrease in agglutinability of treated cells is observed if the incubation is continued up to 4 hours or if the level of enzyme is increased from 40 international units (IU) to 120 IU per 0.5 ml of packed erythrocytes. At higher levels this enzyme causes complete cell lyses within 2 hours possibly due to mellitin contamination. Phospholipase A_2's from Naja mocambique(A_2 NM) and porcine pancrease(A_2PP) have also been tested. The A_2PP has no effect upon D antigen expression whereas the A_2NM is slightly more effective than the bee venom enzyme and can be used at higher levels - up to 360 IU thus far, without lysing the cells during treatment.

In conjunction with these studies we are testing the effect upon D antigen expression of known membrane perturbants such as phenol and diamide. The latter is known to oxidize membrane sulfhydryl groups to disulfide bonds resulting in the reversible cross-linking of spectrin monomers and enhancement of transbilayer mobility of phospholipids such as phosphatidylethanolamine and phosphatidylcholine. Phenol when used alone has no effect upon the expression of D antigenicity and does not significantly reduce the agglutination titer of cells previously treated with phopholipase A_2 . Also prior treatment with phenol appears not to facilitate the action of phospholipase A_2. Diamide on the other hand will reduce D antigen expression yielding agglutination titers at 1:2 of zero. This effect occurs at shorter times when the cells are treated with a combination of diaminde (10mM) and phenol 20mM).

We will continue studying the effect of phospholipases upon the expression of D antigenicity particularly the action of the phospholipase C type enzymes alone and in concert with A_2 phospholipases we have been examining. Combinations of lipases may be useful since their specificities for non-lytic degradation of different membrane phospholipids vary as well as their ability to interact with intact erythrocytes. For example sphingomyelinase C in contrast to phospholipase A_2 cleaves sphingomyelin

into phosphorylcholine which may be released into the aqueous phase while ceramide remains in the cellular membrane. These enzymes will also be used with buffers containing water soluble polymers such as dextran since such compounds have been reported to interact with membrane lipids and so might facilitate enzyme action. It may be that changes in the composition of the erythrocyte membrane produced as a result of the degradion of phospholipids by these lipases will make the D antigen accessable to the action of proteolytic enzymes. We will determine this using proteases such as trypsin and chymotrypsin which have narrow specificities in order to avoid large scale random cleavage of proteins from the cell surface and the production of polyagglutinable cells.

As a complement to the enzyme studies we will continue our exploration of the effect of various membrane modulators or perturbants upon D antigen expression. Since diamide plus phenol reduces D antigenticity it will be of interest to determine the effect of lipase and possibly protease - lipase treatment upon such modified cells. Although preliminary results indicate that incubation of phospholipase A_2 treated cells with a high citrate containing buffer yields a zero agglutination titer with anti D antiserum a great deal of cell lyses occurs during treatment. We wil vary the citrate concentration, buffer pH, time, temperature, etc, in order to determine if it is possible to reduce cell destruction and still produce non-agglutinable cells. Other approaches will also be taken such as altering the cholesterol/phospholipid molar ratio of the red cell membrane and determining if this enhances the enzymatic reduction of D antigenicity. Other membrane modulators such as diethylpyrocarbonate will be tested and if excessive cell damage does not occur they will be used alone and in combination with the approaches previously described.

Characterization of the D Antigen

We have also begun to characterize the D antigen in order to determine its structure so that we can use such information to help formulate ways of modifying its expression. In this study the surface proteins of intact erythrocytes are labeled with I^{125} then the washed cells are reacted with anti Rh D IgG antibody. After lyses of the cells and solubilization of the membrane in non-ionic detergent the immune complex is isolated by absorbtion to Protein-A sepharose, eluted with sodium dodecyl sulfate (SDS) and subjected to SDS-PAGE electrophoresis. Results as shown in figure 1 indicate the presence of a major labeled protein band having an approximate molecular weight of 28-32 kilodaltons. This protein has been reported by several other workers who believe it to be the primary D antigen. Radioactivity is also present in the high molecular weight region of the gel which may at least be partly due to non-specific aggregation of various membrane proteins with the immune complex. But since some of this radioactivity is located in the Band 3 region of the gel and this protein is thought by some workers to contain the D antigen we are currently examining this possibility by comparing the tryptic map profiles or "fingerprints" of the 28-32 Kd protein and Band 3. Such a comparison is shown in figure 2. Band 3 was isolated by subjecting solubilized red cell membranes to SDS-PAGE and cutting the separated Band 3 region from the gel. The 28-32 Kd protein was obtained in the same manner following SDS-PAGE of an anti Rh D immune complex prepared as described above and shown in figure 1 except that no

prior labeling of membrane proteins was performed. The gel slices containing both Band 3 and 28-32 Kd proteins were then labeled with I^{125} and digested with trypsin. The digests were subjected to electrophoresis in one dimension and then chromotography in the second to yield peptide maps or fingerprints characteristic for each protein. As can be seen from this initial experiment the resulting distribution of tryptic peptides of Band 3 (fig 2A) are quite different from that of the 28-32 Kd protein (fig 2B) indicating that the putative D antigen is not derived from Band 3. Further peptide mapping will be performed to confirm these results with Band 3 and possibly other membrane proteins will also be examined if necessary to establish that the 28-32 Kd protein is an integral entity and not derived from another membrane protein.

We are also currently isolating sufficient amounts of 28-32 Kd protein in order to prepare antibodies to it which will be tested against D+ cells and thus confirm its identify as the D antigen. Following that, amino acid and end group analysis of the D antigen will be performed to provide us with the information necessary for attempting to chemically modify its expression in vitro which we would then hope to translate to procedures for the inactivation of D antigenicity on the surface of the intact erythrocyte.

Goals for 1985 - 86

For the forthcoming year we will continue our measurement of the effect of various membrane perturbants upon the expression of D antigenicity. We plan to complete studies of the action of phospholipases upon the D antigen before and after treatment of the red cells with various membrane perturbants. We will also continue our biochemical characterization of the 28-32 Kd protein and confirm its identity as the primary D antigen.

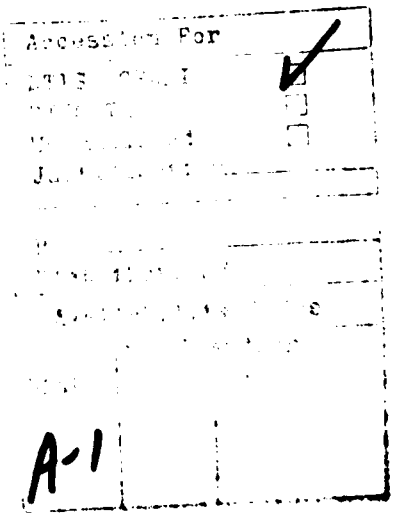

Figure 1

Autoradiogram of:

Lane A isolated immune complex after reacting Rh_0 (D) negative I^{125} labeled cells with anti D antibody.

Lane B- isolated immune complex after reacting Rh_0 (D) positive I^{125} labeled cells with anti D antibody.

Region 1. Aproximate mol wt of 28-32 Kd - believed to be primary D antigenic protein. Present only in lane B - immune complex from D+ cells.

Region 2, 3. Approximate mol wts of 50,000 and 68,8000 Kd respectively - present in trace amounts in immune complex from D negative cells (lane A) as well as in D+ cells.

Region 4. Approximate mol wt of 95,000 known as Band 3 - present in varying amounts in immune complex from D negative cells as well as in immune complex from D+ cells.

Region 5. Approximate mol wt of 200,000 - represents protein aggregates of either Band 3 or glycophorin.

Figure 2

Peptides Maps of:

A: Tryptic digest of Band 3

B: Tryptic digest of 28-32 Kd protein (D antigen)

E: Electrophoresis

TLC: Thin layer chromotography

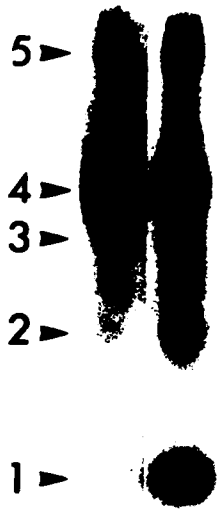

Figure 1

Figure 2

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